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ORIGINAL ARTICLE

Transcriptional Regulation of Arylalkylamine-*N*-Acetyltransferase-2 Gene in the Pineal Gland of the Gilthead Seabream

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Pineal serotonin-*N*-acetyltransferase (arylalkylamine-*N*-acetyltransferase; AANAT) is considered the key enzyme in the generation of circulating melatonin rhythms; the rate of melatonin production is determined by AANAT activity. In all the examined species, AANAT activity is regulated at the post-translational level and, to a variable degree, also at the transcriptional level. Here, the transcriptional regulation of pineal *aanat* (*aanat2*) of the gilthead seabream (*Sparus aurata*) was investigated. Real-time polymerase chain reaction quantification of *aanat2* mRNA levels in the pineal gland collected throughout the 24-h cycle revealed a rhythmic expression pattern. In cultured pineal glands, the amplitude was reduced, but the daily rhythmic expression pattern was maintained under constant illumination, indicating a circadian clock-controlled regulation of seabream *aanat2*. DNA constructs were prepared in which green fluorescent protein was driven by the *aanat2* promoters of seabream and Northern pike. *In vivo* transient expression analyses in zebrafish embryos indicated that these promoters contain the necessary elements to drive enhanced expression in the pineal gland. In the light-entrainable clock-containing PAC-2 zebrafish cell line, a stably transfected seabream *aanat2* promoter-luciferase DNA construct exhibited a clock-controlled circadian rhythm of luciferase activity, characteristic for an E-box-driven expression. In NIH-3T3 cells, the seabream *aanat2* promoter was activated by a synergistic action of BMAL/CLOCK and orthodenticle homeobox 5 (OTX5). Promoter sequence analyses revealed the presence of the photoreceptor conserved element and an extended E-box (i.e. the binding sites for BMAL/CLOCK and OTX5 that have been previously associated with pineal-specific and rhythmic gene expression). These results suggest that seabream *aanat2* is a clock-controlled gene that is regulated by conserved mechanisms.

Key words: melatonin, AANAT, OTX5, BMAL, CLOCK.

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Endogenous circadian oscillators that drive daily rhythms of physiological and behavioural processes exist in all organisms (1). The core molecular mechanism of the circadian oscillator involves intracellular autoregulatory transcriptional/translational feedback loops, which include positive and negative transcription factors. Typically, the positive proteins bind to a core DNA element, E-box, to activate transcription, whereas negative elements suppress this activity (2–4). This mechanism drives the rhythmic expression of clock-controlled genes (4, 5) and ultimately leads to diverse behavioural and physiological rhythms.

Rhythmic production and secretion of melatonin from the pineal gland, peaking at night, constitutes an important component of the

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endogenous clock system in vertebrates. This melatonin rhythm is thought to synchronise other circadian rhythms and to modulate photoperiodic regulation of seasonal physiological rhythms (6). Melatonin rhythms are generated by changes in the activity of serotonin-*N*-acetyltransferase (arylalkylamine-*N*-acetyltransferase; AANAT) in the pineal gland (7). Increased production of melatonin during the night reflects increased AANAT activity, and termination of melatonin production during the day reflects proteasomal degradation of the enzyme (8). Whereas the post-translational regulation of AANAT appears to be ubiquitous, the degree of transcriptional regulation varies among the studied species (9). In rodents, pineal *aanat* mRNA levels exhibit an over 100-fold rhythm (10, 11) whereas, in sheep and monkey, pineal *aanat* mRNA rhythms are absent or maintain a very low (1.5- and 3- fold) amplitude (12–15). Thus, rhythmic *aanat* transcription, AANAT activity, and hence melatonin production, are driven by an internal circadian clock and by external light signals (7, 9, 16–18).

In fish, the melatonin rhythm has been shown to play a role in the regulation of photo-behavioural responses, behavioural thermoregulation, daily activities, body colouration and timing of reproduction (19). As is the case in all nonmammalian vertebrates, the fish pineal gland is photoreceptive and, in most cases, contains an intrinsic circadian clock that drives melatonin rhythms (20). Teleost fishes contain two *aanat* genes: *aanat1* is expressed only in the retina whereas *aanat2* expression predominates in the pineal gland (21, 22). The expression pattern of *aanats* in fish is variable and species-dependent. For example, pike and zebrafish exhibit a clock-controlled rhythm in retinal *aanat1* and pineal *aanat2* mRNA levels (23–26) whereas, in trout, retinal *aanat1* mRNA is rhythmic under light/dark conditions, but not constant darkness, and pineal *aanat2* mRNA levels are constant throughout the 24-h cycle regardless of the light conditions (23, 27, 28).

The gilthead seabream (sb, *Sparus aurata*) has captured the attention of comparative endocrinologists for a number of reasons. First, it is a protandrous hermaphrodite; all individuals reach puberty as functional males and, in later years, undergo sex reversal to become functional females. Second, females have an asynchronous ovarian development and undergo daily cycles of final oocyte maturation, ovulation and spawning during their 3-month breeding season. Third, it is a commercially important aquaculture species. Fourth, the seabream is a member of the perciformes, the largest order of vertebrate, comprising over 7000 species found in almost all aquatic environments.

Melatonin rhythms in the gilthead seabream were shown to be driven by an intrinsic pineal circadian oscillator and to be influenced by photic signals (29, 30). As is the case in all the studied species, light inhibits pineal melatonin production by inducing proteasomal degradation of AANAT and a cAMP-dependent pathway protects AANAT2 from degradation (31). The relative role of *aanat2* transcription in the melatonin rhythm is currently unknown.

In the present study, the transcriptional regulation of seabream *aanat2* was investigated. Analysis of *aanat2* mRNA levels *in vivo* and in cultured pineal glands revealed a low-amplitude rhythm. Functional analysis of the cloned *aanat2* promoter *in vivo* indicates that it is sufficient to drive pineal-specific expression. Analyses in cell systems suggest that this promoter drives rhythmic transcription, mediated by the synergistic activity of the BMAL/CLOCK heterodimer and the homeobox factor OTX5.

Materials and methods

Animal maintenance and sampling

Adult gilthead seabream were maintained as previously described (32). Prior to tissue sampling, fish were anaesthetised in clove oil (33) and decapitated in accordance with the guidelines of the Animal Care Welfare Committee of the Institute of Animal Science, Agricultural Research Organization of Israel.

To determine the temporal expression pattern of *aanat2* under natural light conditions, pineal glands were collected from adult seabream (average weight 250 g) at 3-h intervals throughout the 24-h cycle (six pineal glands/time point). The glands were immediately frozen on dry ice and stored at −80 °C until analysis of *aanat2* mRNA levels. To determine whether *aanat2* transcription is regulated by a pineal-intrinsic circadian oscillator, *aanat2* mRNA levels were monitored in cultured pineal glands. Pineal glands were collected during the morning hours (ZT 4–8) from adult seabream (average weight 400 g) and placed in static culture under a 12 : 12 h light/dark (LD) or continuous light (LL) cycle. Culture conditions were as previously described (29, 30). Starting at ZT 0 of the following day, cultured pineal glands were collected at 3-h intervals for 30 h (six pineal glands per time point for each light treatment), frozen on dry ice and stored at −80 °C until analysis of *aanat2* mRNA levels.

Real-time polymerase chain reaction (PCR) quantification of pineal *aanat2* mRNA

Expression of seabream *β-actin* and *aanat2* in the pineal glands was determined at the transcript level using quantitative PCR assays. Total RNA was extracted using EZ-RNA isolation reagent (Biological Industries, Beit Haemek, Israel) according to the manufacturer's instructions. The mRNA (1 μg) was reverse transcribed using Oligo(dT) primer and MMLV reverse transcriptase (Promega, Madison, WI, USA). Transcript levels were determined by real-time PCR using the ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Triplicate or duplicate first strand cDNA aliquots (5 μl of 1 : 125 diluted) from each sample served as templates in PCR using master mix, SYBR Green I fluorescent dye (Applied Biosystems) and 500 nM gene-specific primers. Amplification reactions were carried out under the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The copy number was determined by comparing critical threshold values with those of recombinant plasmid standards ($0.05–0.5 \times 10^{-6}$ fmol) containing the cDNA inserts and was normalised to the amount of *β-actin* mRNA in each sample. Data for the normalised transcript levels of *aanat*2 are presented as means ± SE (Fig. 1). Data were analysed using ANOVA; specific comparisons were performed using Tukey's post-hoc tests.

Isolation of seabream *aanat2* promoter and promoter-reporter constructs

The 5′-flanking region of seabream *aanat2* was isolated using the universal GenomeWalker Kit (Clontech, Oxford, UK) and *sbaanat2*-specific primers, according to the manufacturers' instructions. The amplified fragment, containing 15 bp of coding sequence, 220 bp of 5′-UTR and 1387 bp of upstream region, was subcloned into pGEM-T-Easy vector and the resulting clone, *pGEM-sb2promt*, was sequenced.

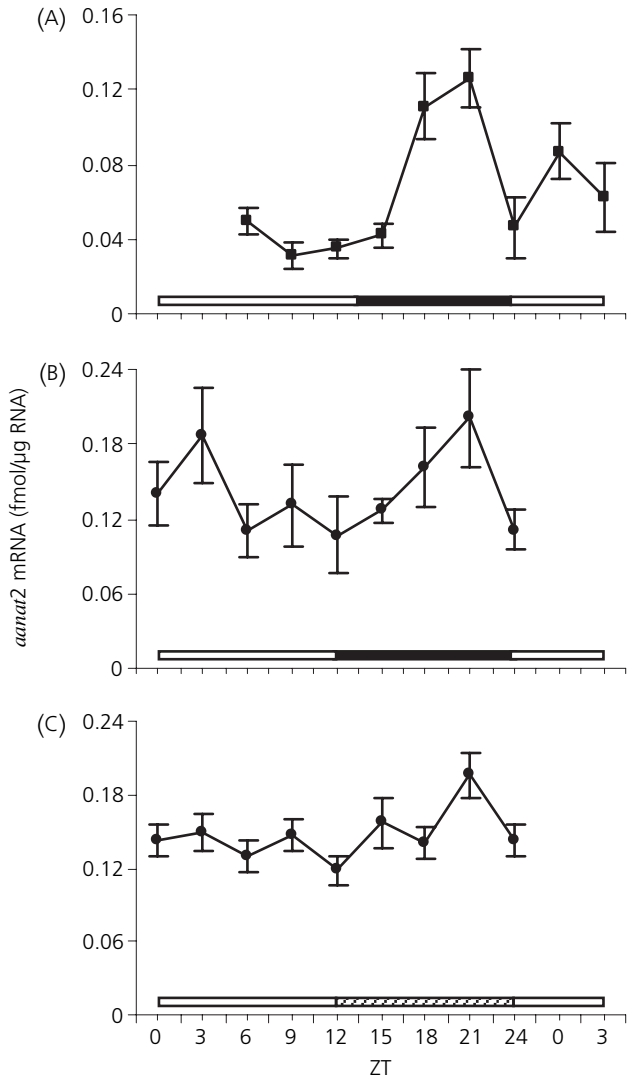

Fig. 1. Daily expression pattern of seabream *aanat2*. The levels of *aanat2* transcripts in seabream pineal glands were measured using quantitative polymerase chain reaction and normalised relative to the levels of *β-actin* transcripts. For the *in vivo* experiment (A), pineal glands of six seabreams were sampled every 3 h, for 27 h, starting at ZT 6. For the *in vitro* experiment (B,C), six cultured pineal glands were sampled every 3 h, for 27 h starting at ZT 0. Data for normalised transcript levels of *aanat2* are presented as means ± SE. Values were compared by ANOVA and Tukey's post-hoc tests.

A fragment containing the upstream region and 5′-UTR (total of 1588 bp) was PCR amplified using a proofreading enzyme DyNAzyme EXT (Finnzymes, Espoo, Finland), *pGEM-sb2promt* as a template and a set of specific primers, sbnat2f1 (5′-CGTG*GTCGAC*GGCCCGGGCTGGTCT-3′) and sbnat2r1 (5′-CGC*GGATCC*GATGTCTCAATAACACAAGAATAGG-3′), containing *Sal*I and *Bam*HI restriction sites (in italics), respectively. The PCR product was double-digested with *Sal*I and *Bam*HI and ligated into *Sal*I/*Bam*HI-cut enhanced green fluorescent protein (EGFP) reporter vector (*pEGFP-1*, Clontech), giving rise to *sbaanat2-EGFP*. This plasmid was sequenced and used in transient expression assays *in vivo* in zebrafish embryos.

A 592-bp fragment was PCR amplified as described above using sbnat2f (5′-CTA*GCTAGC*ACGATCACAAGAAGATTTTC-3′) and sbnat2r (5′-GGA*AGATCT*GATGACTCAATAACACAAGA-3′) primers, containing *Nhe*I and *Bgl*II restriction sites (in italics), respectively. The PCR product was double-digested with *Nhe*I and *Bgl*II and ligated into *Nhe*I/*Bgl*II-cut luciferase reporter vector (*pGL3*, Promega), giving rise to *pGL591sb2*. This plasmid was sequenced and used in a circular form and a linear form to transfect NIH-3T3 and PAC-2 cells, respectively.

Promoter analysis

In vivo *expression assay*

Zebrafish embryos were microinjected with *sbaanat2-EGFP* as previously described (34); in some experiments, plasmids were coinjected with Morpholino-modified antisense oligonucleotides directed against zebrafish OTX5 (OTX5 MO). EGFP expression pattern, pineal-specific, ectopic or both, was determined under a fluorescent microscope and results were subjected to chi-square analysis.

Transient transfection in vitro

Transient transfection assays in NIH-3T3 cells were performed as previously described (35). Briefly, NIH-3T3 cells were plated and transfected with 10 ng *pGL591sb2* and 0.75 µg of a 1 : 1 : 1 mixture of mouse CLOCK (mCLOCK), hamster BMAL1 (hBMAL1) and zebrafish OTX5 expression vectors or empty vector pcDNA and luciferase activity was measured. Data were subjected to two-way ANOVA analysis. Results are the mean of at least three independent experiments each performed in triplicate.

Stable transfection in vitro

PAC-2 cells (36) were cultivated and stably transfected with linearised *pGL591sb2* as previously described (26, 37, 38). Luciferase activity was continuously monitored and data were analysed as previously described (26). Six independent stable transfections were made and assays were performed at least three times. Each well was counted for 3 s at intervals of approximately 1 h. Plates were counted in an uninterrupted cycle. During the light phase, plates were illuminated between counting with a tungsten light source (20 $\mu W/cm^2$). Data were imported into CHRONO (Till Roenneberg, University of Munich, Munich, Germany) and EXCEL (Microsoft Corp., Redmond, WA, USA) using the 'Import and Analysis' macro (S. Kay, Scripps Research Institute). The period estimate was made by linear regression after peak finder analysis with CHRONO, measured under continuous darkness (DD).

Results

Low amplitude seabream *aaanat2* mRNA rhythm

The daily expression pattern of *aanat2* in the pineal gland was determined *in vivo* under natural photoperiodic conditions. This analysis revealed a significant ($P < 0.01$, by ANOVA) effect of ZT on *aanat2* mRNA levels (Fig. 1A). The highest levels, measured at ZT 18 and 21, were 3.3-fold higher than the lowest levels, measured at ZT 9 ($P < 0.05$, Tukey's test). This low-amplitude rhythm is reminiscent of that seen in sheep and monkey, where the pineal *aanat* mRNA levels increased only 1.5- and 3-fold, respectively (7, 12, 13).

To test whether this rhythm is driven by a circadian oscillator, the temporal expression pattern of *aanat2* was monitored *in vitro* under LD and LL. The results revealed a reduction of both LD and

LL amplitudes as compared to that seen in the *in vivo* experiment (Fig. 1A–C). Under both LD and LL, *aanat2* mRNA levels at ZT 21 were approximately two-fold higher than the levels measured at ZT 12. A statistically significant ($P < 0.05$, by ANOVA) effect of the circadian time was found under constant light conditions (LL) (Fig. 1C), providing evidence for clock regulation.

Conserved regulatory elements in the seabream *aanat2* promoter

Analysis of the proximal 5′ regulatory region of the seabream *aanat2* revealed several putative transcription factor binding sites (Fig. 2). One canonical E-box element (CACGTG) and an imperfect element were found at nucleotides 71–76 and 105–110 upstream of the transcription start site, respectively. The location of the canonical E-box is at approximately the same position in which E-box elements are present in the *aanat2* gene of zebrafish and northern pike (Fig. 2). In zebrafish *aanat2*, these elements were shown to be functional (26). Multiple copies of the photoreceptor conserved element (PCE, TAATT/C) are present upstream to the E-box (Fig. 2). In zebrafish, this element recruits OTX5 and mediates the pineal-specific expression of *aanat2* (26, 34, 35, 39).

The seabream *aanat2* promoter drives pineal-specific expression

In zebrafish, pineal-specific expression of *aanat2* begins at 22 h postfertilisation (hpf) (25). This is determined by the activity of a photoreceptor-specific homeodomain, OTX5, and mediated by PCEs located within a downstream regulatory region (PRDM) (35) and the promoter (26). Towards investigating the generality of this mechanism, the ability of the seabream *aanat2* promoter to drive pineal expression was tested *in vivo* in zebrafish embryos by microinjection of *sbaanat2-EGFP*.

Injection of the *sbaanat2-EGFP* resulted in EGFP expression in 70% of the injected embryos ($n = 112$). Remarkably, among these EGFP-positive embryos, a significant number (66%, $P < 0.001$ by chi-square analysis) exhibited a signal in the pineal gland (Fig. 3), of which 20% exhibited a restricted signal (Fig. 3A,B). Ectopic expression of EGFP without any pineal expression was observed in 34% of the EGFP-positive embryos. Similar results were obtained after microinjection of a DNA construct in which EGFP is under the control of the pike *aanat2* promoter. These results indicate that the seabream and pike *aanat2* promoters contain the required elements for pineal expression.

To determine whether pineal expression requires OTX5 action, *sbaanat2-EGFP* was microinjected along with OTX5 MO. Half of the embryos were EGFP-positive. However, pineal expression did not occur in embryos that were coinjected with OTX5 MO; essentially all EGFP-positive embryos showed ectopic expression only. Thus, knockdown of OTX5 completely blocked pineal expression of the seabream *aanat2* promoter, suggesting that OTX5 may act through the seabream *aanat2* promoter to enhance pineal expression of this gene.

Seabream *aanat2* promoter activity is driven by a circadian oscillator

Promoter activity was tested by means of stable transfection in the PAC-2 zebrafish cell line, which contains a light entrainable clock (38). Cells were stably transfected with *pGL591sb2* in which luciferase is driven by the seabream promoter. Expression was tested by exposure of the transfected cells to LD cycles followed by DD and then reversed light/dark cycles (DL).

The *aanat2* promoter drove a rhythm of expression under LD conditions with a peak of luciferase activity at ZT 18.9 ± 0.9 as determined by peakfinder analysis (Fig. 4 and data not shown). Cycling expression was also maintained for six days under DD with a free-running period length of 25.96 ± 1.2 h (Fig. 4 and data not shown). When cells were then re-exposed to an LD cycle shifted by approximately 12 h relative to the phase of the free running rhythm (DL) conditions, the phase of the rhythm gradually shifted to match the new LD cycle within three cycles. These results are consistent with previous observations on the entrainment of expression rhythms directed by E-box enhancer elements within certain promoters including the zebrafish *aanat2* promoter in PAC-2 cells (26, 38). It should be emphasised that not all E-box-containing promoters are rhythmic in PAC-2 or other cell lines. These stable luciferase reporter assays indicate that in the context of PAC-2 cells, the seabream *aanat2* promoter is controlled by the endogenous circadian oscillator.

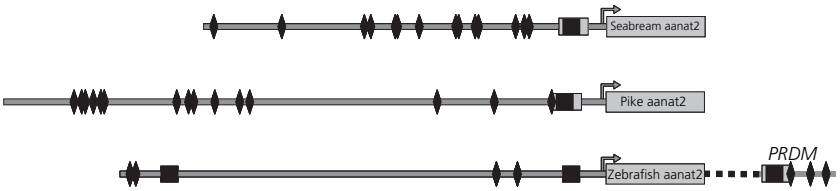

Fig. 2. Regulatory elements in the seabream *aanat2* promoter. The relative location of E-box (squares) and photoreceptor conserved element (diamond) elements in the seabream (accession number DQ887564), zebrafish (accession numbers AF494081 and AY380805) and pike (accession number DQ887565) *aanat2* regulatory regions are shown. The E-box elements in seabream and pike *aanat2* promoters and, in the downstream regulatory region (PRDM) of zebrafish, *aanat2* are within a 13-bp repetitive motif (grey rectangle). PRDM is located approximately 4 kb downstream to the transcribed sequence. An arrow marks the start site of transcription.

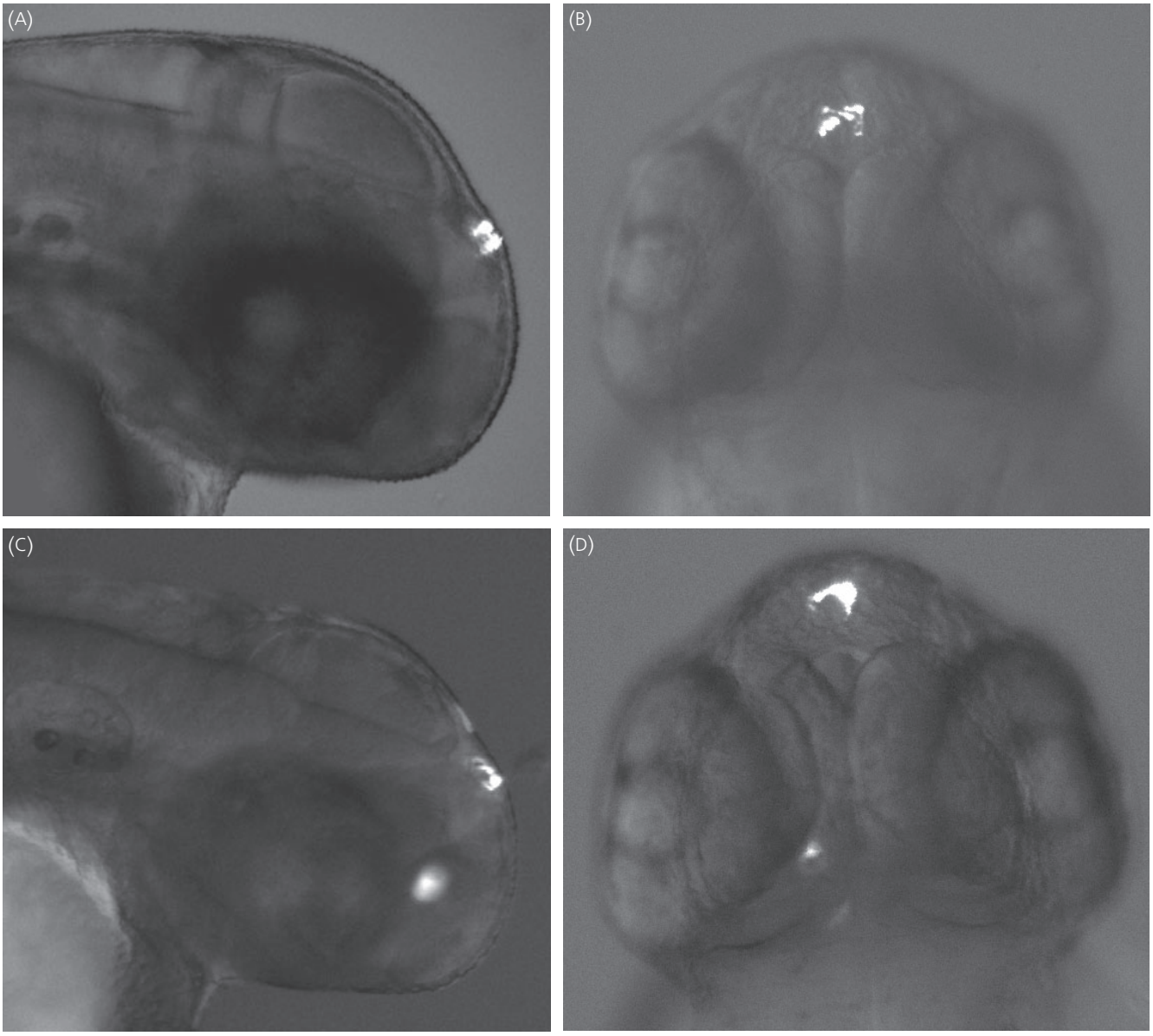

Fig. 3. The seabream *aanat2* promoter drives enhanced expression in the pineal gland. Lateral (A,C) and dorsal (B,D) views of representative 3-day-old zebrafish embryos exhibiting pineal-specific (A,B) and pineal with ectopic (C,D) enhanced green fluorescent protein expression.

Seabream *aanat2* promoter activity is controlled by BMAL/CLOCK and OTX5

Previous findings indicated that OTX5 and BMAL/CLOCK bind to PCEs and E-box elements, respectively, located within the regulatory regions of zebrafish *aanat2*. Pineal-specific and rhythmic expression are regulated by a synergistic interaction of these two complexes (26, 35). To investigate whether this mechanism is universal, the effect of these factors on the activity of the PCE- and E-box-containing seabream *aanat2* promoter was tested. *pGL591sb2* was co-transfected into NIH-3T3 cells with either an empty vector (pcDNA) or mixtures of mBMAL/hCLOCK and OTX5 expression vectors as previously described (35). Co-transfection of the promoter-reporter constructs with OTX5 increased reporter gene expression over control levels by approximately five-fold ($P < 0.001$, Fig. 5). Co-transfection with BMAL/CLOCK did not increase reporter gene expression over control levels. Nevertheless, co-transfection with both hBMAL/mCLOCK and OTX5 resulted in an eight-fold ($P < 0.02$) increase in luciferase activity (Fig. 5), indicating that BMAL/CLOCK and OTX5, possibly through the E-box and PCE elements, synergistically enhance the activity of the seabream *aanat2* promoter.

Discussion

Photoreceptors of the pineal gland and retina are evolutionarily and developmentally linked. They share many proteins that play a

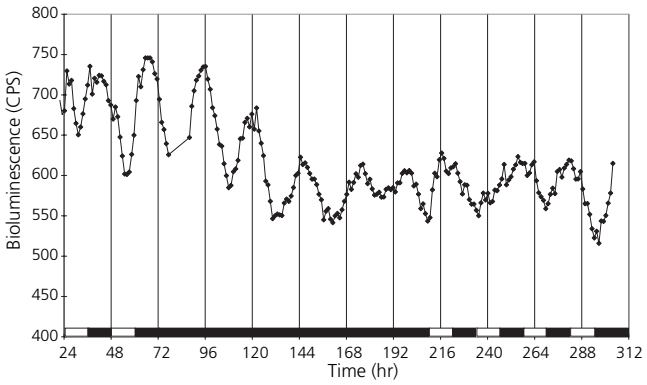

Fig. 4. Circadian rhythms of seabream *aanat2* promoter activity. Representative bioluminescence assay of pools of PAC-2 zebrafish cells stably transfected with a *sbaanat2* promoter-luciferase reporter and monitored for 12 days under altered light conditions (a white/black bar shows the light and dark periods, respectively). The mean bioluminescence values calculated from eight wells is plotted on the *y*-axis (counts per second) and hours on the *x*-axis. Cells maintained for 3 days in light/dark (LD) under 24-h cycles (12 : 12 h), transferred to continuous darkness (DD) for 6 days and then subjected to a reversal of the phase of the LD cycle (DL) for an additional 4 days. Data are plotted starting 24 h after the start of the first LD period. A gap of 10 h in the plot between 77 and 87 h represents an interruption in the experiment for treatment of another plate on the counter. During this interruption, the plates were maintained in DD.

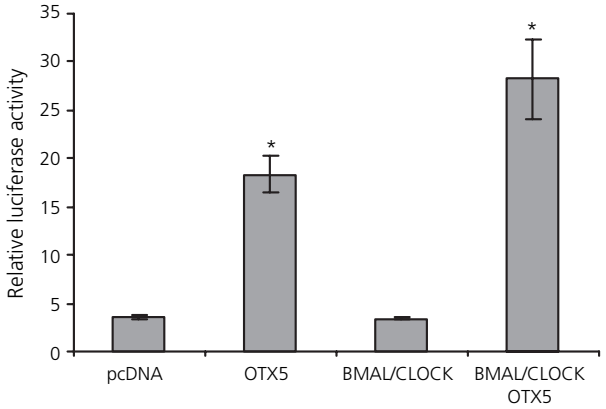

Fig. 5. BMAL/CLOCK and OTX5 activation of the seabream *aanat2* promoter. NIH-3T3 cells were co-transfected with combinations of a luciferase reporter driven by *aanat2* promoter construct and expression vector of OTX5 and hBMAL/mCLOCK. Transcriptional activity is expressed as relative luciferase activity (mean ± SE). Statistical analysis was performed by two-way ANOVA.

role in photoreception, phototransduction, and melatonin production (40). In teleost fish, whole genome duplication and subsequent mutations in the duplicated genes (41) have resulted in two *aanats* with distinct functions and expression patterns: *aanat1* is involved in paracrine functions in the retina whereas *aanat2* has an endocrine function in the pineal gland (21, 22, 24).

In zebrafish, pineal expression of *aanat2* is controlled by the photoreceptor-specific homeodomain protein, OTX5. Consistently, expression of *aanat2* in the pineal gland is reduced by OTX5 knockdown (35, 39). This action is mediated by PCEs located within the promoter and PRDM (26, 35). In the present study, *in vivo* promoter analyses indicated that the *aanat2* promoters of seabream and pike are able to drive pineal expression. Furthermore, seabream *aanat2* promoter activity was abolished *in vivo* in OTX5-knock-down embryos and was augmented *in vitro* by OTX5 in NIH-3T3 cells. Consequently, the transcriptional regulation previously described only in zebrafish may now be extended to seabream, *and* also possibly to other fish *aanat2* genes. One difference found in the present work was the fact that seabream and pike promoter regions alone were sufficient to drive pineal-specific expression, in contrast to zebrafish, where both the promoter and PRDM were required for pineal-specific expression of *aanat2* (34, 42). It is tempting to speculate that the large number of PCEs in the seabream and pike *aanat2* promoters account for this enhanced pineal expression; however, this is probably not the case because the zebrafish *aanat1* promoter, which also contains a larger number of PCEs, is inactive in the pineal gland (34). Notably, a motif discovery tool (MEME) (43) has identified a 13-bp motif that is present in the seabream and pike *aanat2* promoters (Fig. 2) and in the PRDM but not in the zebrafish *aanat2* promoter; interestingly, this motif includes the E-box (Fig. 2). Future comparative analysis of the seabream and other fish *aanat2* promoters and the zebrafish PRDM may shed some light on this and other elusive pineal-specific elements.

The present study also demonstrates a rhythmic expression pattern of seabream *aanat2*. This was shown in the pineal gland both *in vivo* and, to a lesser extent, in culture. Support for the involvement of the core circadian oscillator in the regulation of seabream *aanat2* comes from the rhythmic expression driven by its promoter in stably transfected PAC-2 cells. This expression pattern resembles that of other E-box-containing clock-driven promoters (26, 38). Furthermore, activity of the seabream *aanat2* promoter was enhanced by BMAL/CLOCK in NIH-3T3 cells. Accordingly, seabream *aanat2* may be considered as a clock-controlled gene.

Studies in zebrafish have provided several lines of evidence that the mechanisms underlying tissue-specificity of *aanat2* are intertwined with those that determine rhythmicity. First, knockdown of OTX5 reduces the expression of rhythmic pineal genes, including *aanat2*, but does not have an effect on nonrhythmic genes (39). Second, OTX5, and the clock proteins heterodimer, BMAL/CLOCK, bind PCEs and E-box elements located within the *aanat2* promoter and PRDM and activate transcription (26, 35). Moreover, a synergistic effect of OTX5 and BMAL/CLOCK that is affected by the distance between the PCEs and E-box suggests a physical interaction between the two nucleoprotein complexes (35). Generalisation of this novel mechanism, which was shown to date only in zebrafish, calls for further investigation and extension to other species. In the present study, we found that the seabream and pike *aanat2* promoters contain a combination of E-box and PCEs and that activity of the seabream *aanat2* promoter was augmented in the cell system by the combination of BMAL/CLOCK and OTX5. It is therefore possible that the transcriptional mechanisms underlying tissue-specific and rhythmic expression of *aanat2* are conserved among fish.

Noteworthy is the presence of E-Box/PCE combinations in the regulatory regions of chicken and rat *aanats* and other pineal-specific genes (44).

Rhythms of *aanat2* mRNA expression were of relatively low amplitude. This may suggest that post-translational regulation of the seabream AANAT2 (9) has more impact on the melatonin rhythm than the transcriptional regulation. This low amplitude was further reduced when pineal glands were kept in culture. It has been generally accepted that the teleost pineal gland contains an intrinsic circadian oscillator that is independent of any neural regulation. However, this notion has been based on studies in a relatively limited number of fish species. The severely attenuated *aanat2* mRNA rhythm in cultured pineal glands, as compared with the rhythm encountered *in vivo*, may indicate that, in addition to its intrinsic circadian oscillator, the seabream pineal gland is also stimulated by an extra-pineal oscillator, as is the case in birds. Indeed, a comparison of pineal rhythmic *aanat* mRNA expression in the chicken (*Gallus domesticus*) between *in vivo and in vitro* LD experiments, revealed considerably higher amplitudes *in vivo* (ten-fold *in vivo* versus only two- to five-fold *in vitro*) (45, 46). Moreover, in sea bass, another perciform fish, photic information from the eyes was shown to be required for normal secretion of pineal melatonin (47). Thus, the dogma that the circadian clock in the fish pineal gland is absolutely autonomous is subject to question.

In summary, the present study demonstrates that seabream *aanat2* is a pineal-specific, clock-controlled gene; its promoter contains the necessary and conserved elements for driving pineal expression and for mediating the activity of the molecular circadian oscillator in the pineal gland.

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